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The effect of cavity-filling mutations on the thermostability of *Bacillus stearothermophilus* neutral protease

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Cavities in the hydrophobic core of the neutral protease of *Bacillus stearothermophilus* were analyzed using a three-dimensional model that was inferred from the crystal structure of thermolysin, the highly homologous neutral protease of *B.thermoproteolyticus* (85% sequence identity). Site-directed mutagenesis was used to fill some of these cavities, thereby improving hydrophobic packing in the protein interior. The mutations had small effects on the thermostability, even after drastic changes, such as Leu284→Trp and Met168→Trp. The effects on T50, the temperature at which 50% of the enzyme is irreversibly inactivated in 30 min, ranged from 0.0 to +0.4°C. These results can be explained by assuming that the mutations have positive and negative structural effects of approximately the same magnitude. Alternatively, it could be envisaged that the local unfolding steps, which render the enzyme susceptible towards autolysis and which are rate limiting in the process of thermal inactivation, are only slightly affected by alterations in the hydrophobic core.

Key words: *Bacillus*/cavity/hydrophobic/neutral protease/thermostability

Introduction

Hydrophobicity and high quality packing of residues in the protein core are generally accepted to be a major stabilizing factor in proteins (Kauzman, 1959; Tanford, 1980; Dill, 1990; Sharp, 1991). Since very dense packing would require energetically unfavourable perturbations of protein stereochemistry, the core structure of a protein probably represents a compromise between close packing and conformational strain (Conolly, 1986; Karpusas *et al.*, 1989; Creighton, 1991). Therefore, it has been suggested that cavities are an inevitable element of protein architecture (Karpusas *et al.*, 1989). Indeed, cavities have been detected in the cores of most proteins (Rashin *et al.*, 1986). Such cavities are energetically unfavourable because their surrounding residues have less Van der Waals interactions than if these residues were located in a densely packed environment (Pakula and Sauer, 1989).

Site-directed mutagenesis experiments in which hydrophobic core residues were replaced by smaller ones have illustrated that cavity formation and loss of Van der Waals contacts destabilize proteins (Yutani *et al.*, 1987; Kellis *et al.*, 1988; Sandberg and Terwilliger, 1989; Shortle *et al.*, 1990). Similar studies have indicated that, in addition, the spatial arrangement of hydrophobic residues in the core is important for stability (Karpusas *et al.*, 1989; Lim and Sauer, 1989; Sandberg and Terwilliger, 1989). These mutagenesis studies mostly concerned mutations that destabilized the protein. Examples of proteins that were stabilized by mutations aimed at improving packing density are scarce (Matsumura *et al.*, 1988; Karpusas *et al.*, 1989).

The aim of this study was to investigate whether the neutral protease of *Bacillus stearothermophilus* (Npr-ste; Fujii *et al.*, 1983; Takagi *et al.*, 1985; Priest, 1989) could be stabilized by site-directed mutations designed to fill cavities in its hydrophobic core. To this purpose a three-dimensional model of the neutral protease was built on the basis of the known three-dimensional structure of thermolysin (Colman *et al.*, 1972; Matthews *et al.*, 1972; Holmes and Matthews, 1982), which is highly homologous to Npr-ste (85% sequence identity). Inspection of the crystal structure of thermolysin and the model of Npr-ste showed that both enzymes contain several (often interconnected) cavities in their C-terminal domain. This paper describes six different site-directed mutations which were designed to fill cavities that are clustered around Phe267 in the C-terminal domain of Npr-ste, in total comprising a volume of ~50 Å^3 (Figures 1 and 2). The results show that the mutations in the hydrophobic core had positive but small effects on the thermostability of the enzyme.

Materials and methods

Genes, plasmids and site-directed mutagenesis

A 2.35 kb *NcoI–BclI* fragment from plasmid pNP22 (Fujii *et al.*, 1983), containing the *B.stearothermophilus npr* gene, was subcloned in the *BclI* site of the high-copy number *B.subtilis* vector pTZ12 (Aoki *et al.*, 1987), yielding pGE501 (Figure 3). For the subcloning procedure and for production of the enzyme *B.subtilis* strain DB117 (Eijsink *et al.*, 1990) was used. For site-directed mutagenesis (Stanssens *et al.*, 1989) suitable fragments of the *npr* gene were subcloned in the *E.coli* vector pMa/c. For the mutagenesis procedure *E.coli* strains WK6 and WK6mutS (Zell and Fritz, 1987) were used. After mutagenesis the DNA sequence of the mutated gene fragment was verified using the dideoxy chain termination method (Sanger *et al.*, 1977).

Table I. Oligonucleotides used for site-directed mutagenesis[a]

Mutation	Sequence	
M168W	5′-CGAAAATATC**GGACC**ACGCTTCATTG-3′	(*Ava*II)
A240V	5′-GCAAGTACGCCACTTT**ATTAAT**GATGCCG-3′	(*Asn*I)
A241V	5′-GAGCAA**GTAGAC**CGCTTTATTG-3′	(*Acc*I)
L284W	5′-CGCAGGCG**GCGCGC**CACTGGCTGAAG-3′	(*Bss*HII)
C288L	5′-CCGCTTGCA**CCAAGG**CGGCACG-3′	(*Sty*I)
C288I	5′-CGCTTGCA**CGATCG**CGGCACGCAG-3′	(*Pvu*I)

[a]The wild-type sequence of the *B.stearothermophilus npr* gene has been described by Takagi *et al.* (1985). Nucleotide substitutions are shown in bold type face. Restriction sites, inserted to facilitate selection of mutant clones, are shown between brackets and underlined in the sequence.

Subsequently complete genes, encoding mutant neutral proteases were reconstituted in *B.subtilis* DB117. Oligonucleotides used for site-directed mutagenesis are shown in Table I.

Production, purification and characterization of neutral proteases

Production, purification and SDS–PAGE of wild-type and mutant neutral proteases were performed as decribed previously (Van den Burg *et al.*, 1989; Eijsink *et al.*, 1990). After purification the enzymes were stored in elution buffer [20 mM sodium acetate, pH 5.0, 5 mM $CaCl_2$, 2.5 M NaCl, 20% (v/v) isopropanol, 0.03% (w/v) sodium azide] at −18°C. For the determination of thermostability, aliquots of diluted enzyme [0.1 μM in 20 mM sodium acetate, pH 5.0, 5 mM $CaCl_2$, 62.5 mM NaCl, 0.5% (v/v) isopropanol, 0.03% (w/v) sodium azide] were incubated for 30 min at various temperatures. Subsequently, residual activities were determined using a casein assay (Fujii *et al.*, 1983). Residual activities were expressed relative to the initial activity. T50 is the temperature at which 50% of the initial activity was retained. The thermostability of the mutant enzymes is expressed as dT50, being the difference in T50 between the mutant and the wild-type enzyme.

Model building and mutant prediction

The alignment between thermolysin (316 residues) and Npr-ste (319 residues) has been described by Eijsink *et al.* (1990). Npr-ste has three extra residues inserted between residues 28 and 29 in thermolysin. In this paper all residues in Npr-ste are numbered according to the thermolysin amino acid sequence.

The model of Npr-ste, built on the basis of the structure of thermolysin (Holmes and Matthews, 1982), has been described elsewhere (Eijsink *et al.*, 1990, 1992a). Molecular modelling was performed with the program WHAT IF (Vriend, 1990) using an Evans and Sutherland PS390 interactive graphics system and an ESV10 workstation. Cavities were identified with the fast surface detection method of Voorintholt *et al.* (1989), as implemented in WHAT IF (Vriend, 1990). This method gives similar results to the methods described by Conolly (1985) and Rashin *et al.* (1986). The grid spacing was 0.72 Å. The probe diameter was set at 1.5 Å in order to define as a cavity the space in which a covalently bound methyl group could be positioned. Such a methyl group would extend an existing side chain by ~1.5 Å. The volume of a cavity was defined as the volume enclosed by the surface traced out by the probe sphere. Van der Waals radii used were: S, 2.0 Å; N, 1.7 Å; C, 1.8 Å; O, 1.4 Å.

Possible conformations of the side chains of introduced residues were analyzed by searching a protein structure database (206 proteins) for the best-matching 7-residue fragments with the residue of interest in the middle position, using an alpha-carbon distance geometry algorithm similar to that of Jones and Thirup (1986). The side chain of the mutant residue was positioned in the most preferred rotamer that was attainable without the introduction of unfavourable Van der Waals overlaps. Subsequently, the mutant structures were subjected to energy minimization, followed by 2 ps molecular dynamics at 300 K to get out of high energy local minima, and a final, exhaustive energy minimization, using GROMOS (Van Gunsteren and Berendsen, 1987). During these procedures constraints were placed on the alpha-carbon positions to keep them within ~0.25 Å of the original positions. The procedures were carried out using the atomic partial charges incorporated in GROMOS for calculations *in vacuo*.

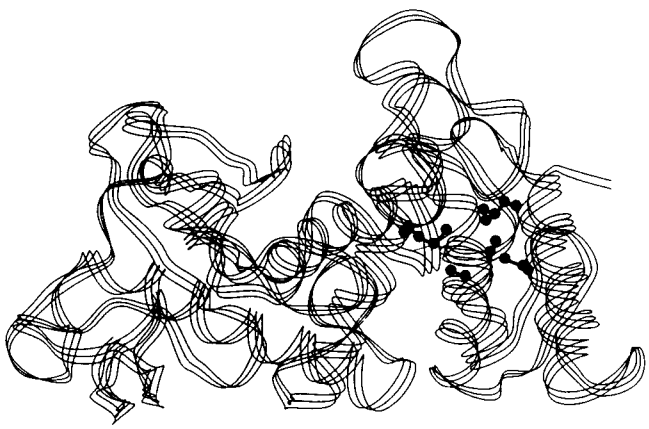


Fig. 1. Ribbon representation of *B.stearothermophilus* neutral protease. The C-alphas and the side chain groups of the mutated residues (Met168, Val240, Val241, Leu284, Cys288) are shown as ball-and-stick models. All five residues are located in alpha-helices.

Results

Structural analysis

Around Phe267 Npr-ste contains a number of (often interconnected) cavities, that are surrounded by ~15 hydrophobic residues (Ala, Val, Leu, Ile, Phe, Met and Cys; Figures 2 and 4). These residues are identical between Npr-ste and thermolysin, except for Met168, Met263 and Cys288, which are replaced by Ile, Leu and Ala, respectively, in the latter enzyme. In the thermolysin crystal structure the cavities shown in Figure 2 are

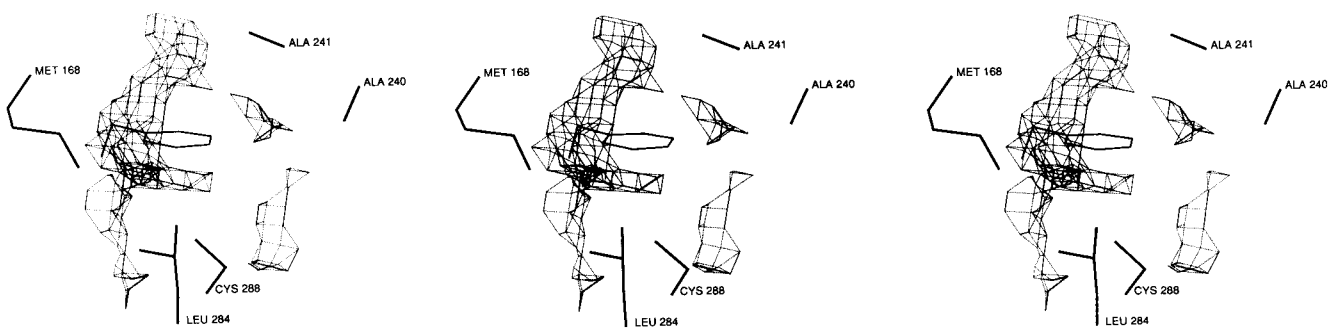


Fig. 2. Cavities around Phe267 in Npr-ste. The side chain of Phe267 is drawn in thin solid lines. The side chains of residues that were mutated are drawn in thick solid lines, with labels positioned at their C-alphas. For clarity, other residues surrounding the cavities were omitted. Cavities were contoured with dotted lines at the surface traced out by the centre of a probe with a 1.5 Å diameter. The contoured areas thus indicate the volume where the centre of this probe can be positioned, without introduction of Van der Waals overlap.

devoid of crystal waters and it was assumed that this also applies to the corresponding cavities in Npr-ste. In the model of Npr-ste the total volume of the cavities shown in Figure 2 is ~50 Å^3.

To verify whether the energy minimization and molecular dynamics procedures that were used in the final stage of model building (Eijsink *et al.*, 1990) could have introduced artifacts with respect to cavities in the Npr-ste model, the X-ray structure of thermolysin was subjected to the same procedures. Because of these procedures, several cavities in the thermolysin structure were changed, indicating that errors indeed occur and that modelled cavities may differ from reality. However, the cluster of cavities around Phe267 remained largely unaltered, showing that in this area the impact of energy minimization and molecular dynamics on the shape and size of cavities was small. On the basis of these observations and because of the fact that most residues around Phe267 are conserved between Npr-ste and thermolysin, it was assumed that the Npr-ste model was sufficiently accurate for adequate modelling of mutations designed to fill the cavities in this region.

Modelling studies suggested that several mutations at each of the five positions shown in Figures 1 and 2 could reduce the size of the cavities. Those mutations were selected that filled a cavity as much as possible, while keeping the number of unfavourable side effects (conformational strain; Van der Waals overlaps) at a minimum. In two cases it appeared that this might be achieved by introducing Trp residues, at positions 168 and 284. Figure 4(A–F), presenting details of the modelled structures of the mutant enzymes, suggests that most of the introduced hydrophobic side chains can be accommodated by the cavities without introducing Van der Waals overlaps. Important structural effects of the mutations are summarized in Table II.

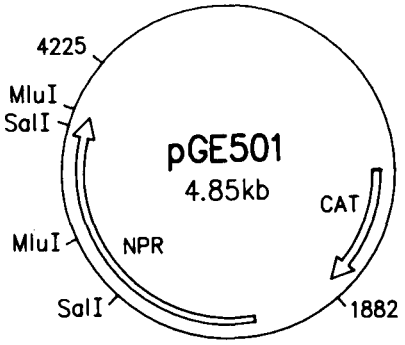


Fig. 3. Map of plasmid pGE501 indicating the chloroamphenicol resistance gene (CAT) and the gene for *B.stearothermophilus* neutral protease (npr). The fragment inserted in the vector pTZ12 runs from 1882 to 4225.

Table II. Characteristics of mutant *B.stearothermophilus* neutral proteases

Mutation	dT50[a]	Salient structural effects[b]
M168W	+0.3	Trp side chain in scarcely populated rotamer (Figure 4A)
A240V	0.0	Val side chain in scarcely populated rotamer; A→V in helix is unfavourable (Figure 4B)
A241V	0.0	Val side chain can adopt most preferred rotamer; A→V in helix is unfavourable (Figure 4C)
L284W	+0.4	Trp side chain can adopt most preferred rotamer (Figure 4D)
C288L	+0.2	Leu side chain can adopt most preferred rotamer; Van der Waals overlap between Cδ1, Cδ2 and side chain of Phe267 (Figures 4E and 5)
C288I	+0.4	Ile side chain can adopt most preferred rotamer; Van der Waals overlap between Cδ and side chain of Phe267 (Figures 4F and 5)

[a]dT50 values were determined as described in Materials and methods; they represent the average value of at least four independent assays; error margins were estimated to be ±0.1°C. The T_{50} of the wild-type enzyme was 68.5°C.

[b]As a result of the mutations, packing density in the core was improved. No Van der Waals overlap was introduced unless stated otherwise. Relevant additional structural features of the mutant enzymes are summarized in this column.

Mutant neutral proteases

Using oligonucleotide-directed mutagenesis, mutant *npr* genes were constructed, encoding the Npr-ste variants listed in Table II. The mutant enzymes were similar to the wild-type enzyme with respect to the level of production by *B.subtilis* DB117 and electrophoretic mobility during SDS–PAGE. After purification, the mutant and wild-type enzymes exhibited similar specific activities towards casein (data not shown). The thermostabilities of the mutant enzymes are indicated in Table II. The data in this table show that the effects of the mutations on thermostability varied from zero to slightly positive.

Discussion

On the basis of model-building of the three-dimensional structure of Npr-ste and mutants thereof, it was expected that the cavity-filling mutations described above would improve the packing density and Van der Waals interactions in the hydrophobic core of this protein, thus increasing its thermostability. Indeed, some of the mutations increased the thermostability of Npr-ste, although their effect was small. Those substitutions that concerned the largest side chains tended to result in the most pronounced increase in thermostability of the enzyme.

To compensate for the uncertainties inherent to the model-building procedures we constructed six different mutations to fill the cavities. Remarkably, none of these mutations destabilized the protein, despite the model-building uncertainties and some obvious negative side effects (listed in Table II). A double mutant, containing Trp at positions 284 and 310 (Leu and Phe in wild-type Npr-ste, respectively) was only slightly destabilized (−0.4°C; V.G.H.Eijsink, O.R.Veltman and B.W.Dijkstra, unpublished observations). The difference in dT50 between the Leu288 and the Ile288 mutants was expected from a comparison of their respective models. As shown in Figure 5, both introduced side chains have Van der Waals overlaps with the side chain of Phe267, which bends away in the model building procedures. Bending is more pronounced in the case of the Leu288 mutant, which, accordingly, has a lower thermostability. Interestingly, Phe267 and Phe310 (Figure 4) have the second and third highest B-factors of all buried residues in the crystal structure of thermolysin (Holmes and Matthews, 1982), indicating that these residues have relatively high conformational freedom. Thus the movement of Phe267 that resulted from the mutations at position 288 is probably only slightly unfavourable, explaining why the Cys288→Leu and Cys288→Ile mutations exerted a stabilizing effect, despite the Van der Waals overlaps.

Site-directed mutagenesis experiments have supported the general view that the hydrophobic core of a protein represents a delicate balance between high packing density and the conformational strain needed to achieve this (Sandberg and Terwilliger, 1989, 1991; Karpusas *et al.*, 1989). It has been shown that a protein can adjust the packing of its hydrophobic core to accommodate a great variety of mutations as long as hydrophobicity is preserved (Lim and Sauer, 1989). However, such mutations often introduce significant negative effects such

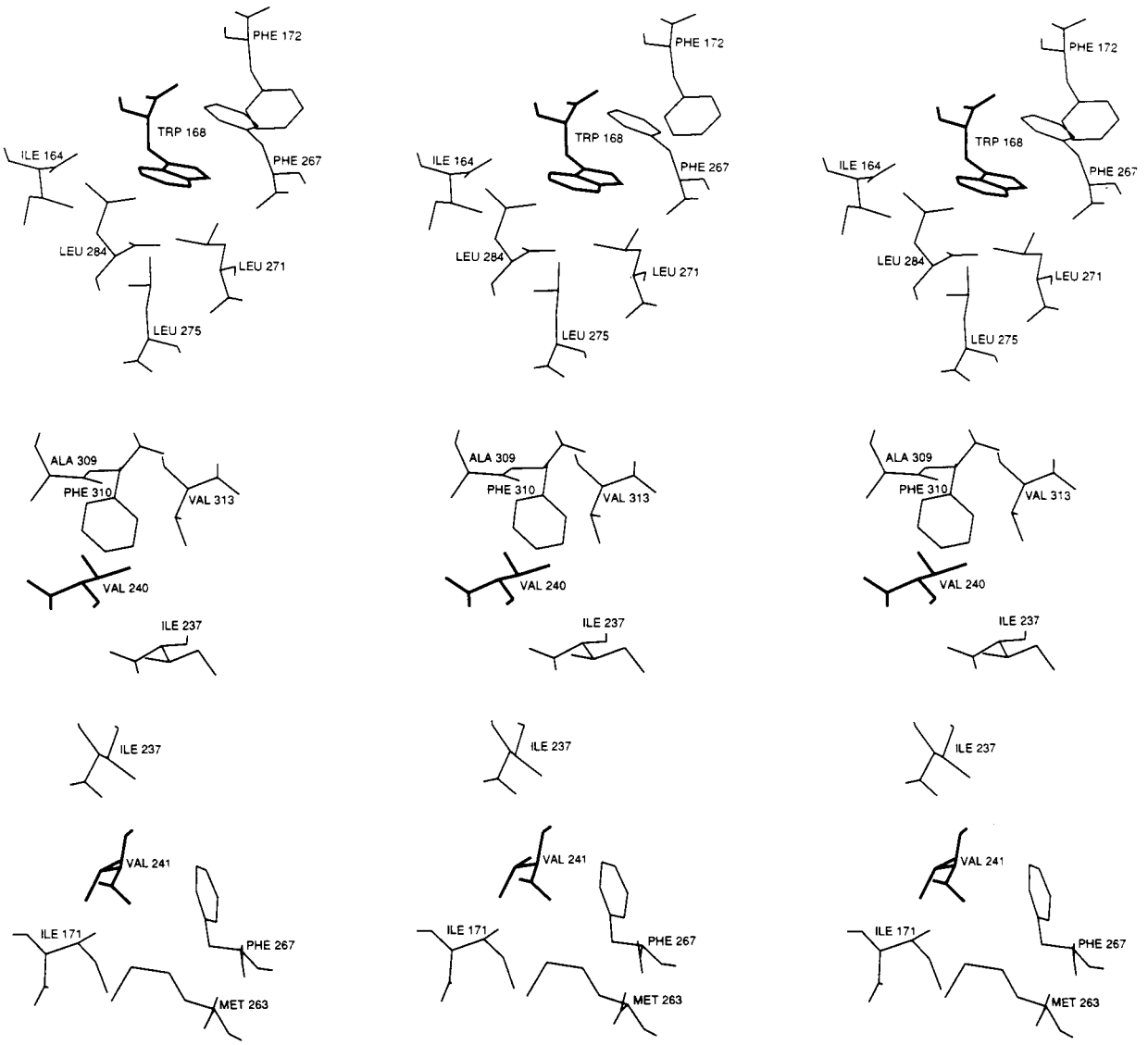
as the introduction of strain and Van der Waals overlaps which destabilize the protein (Karpusas *et al.*, 1989). The insensitivity of Npr-ste towards mutations altering its hydrophobic core could be explained by assuming that positive and negative effects of altering the packing arrangement tend to cancel out (Karpusas *et al.*, 1989). It is, however, doubtful whether such an explanation applies to mutations as drastic as the introduction of one or two Trp residues.

Alternatively, the small effect of mutations in the hydrophobic core of Npr-ste may be explained on the basis of the specific features of the thermal inactivation process of proteolytic enzymes. It has been shown that local unfolding, which renders the enzyme susceptible towards irreversible autolytic degradation, is the rate-limiting step in the thermal inactivation process of Nprs (Eijsink *et al.*, 1991a, 1992b). It is likely that the rate-determining local unfolding steps concern regions at the surface of the protein, the stability of which would only marginally be influenced by mutations in the hydrophobic core.

As a consequence of the latter explanation, it is to be expected that mutations at the surface of the protein will be most effective in stabilizing Npr-ste. This has indeed been observed. Large increments in thermostability were obtained by mutating the solvent exposed residues 63 (dT50 for Thr63Phe = +6.2°C; Van den Burg *et al.*, 1991) and 69 (dT50 for Ala69Pro = +5.5°C; V.G.H.Eijsink, G.Vriend and G.Venema, unpublished results). Mutations at buried positions in Npr-ste had smaller stabilizing effects (in the range of +1.0°C; Eijsink *et al.*, 1991b, 1992a; Vriend *et al.*, 1991).

In order to discriminate between the two explanations for the insensitivity of Npr-ste towards cavity-filling mutations it would be desirable to study reversible global unfolding of the Npr-ste variants and thus measure the energetic effects of the mutations. However, such studies are complicated, if not impossible, because of the occurrence of autolysis (Dahlquist, 1976; Mitchinson and Wells, 1989; Eijsink *et al.*, 1991a). Further insight might be gained by mutations that result in the removal of hydrophobic groups from the protein interior. Their effect would be destabilizing if the first explanation were valid, whereas thermostability would be largely unaffected if the second explanation held.

Rigorous structural analysis of the site-directed mutations is hampered by the lack of crystallographic data for both the wild-type and the mutant neutral proteases. However, previously described data concerning the model-building procedure (Eijsink *et al.*, 1992a) and the engineering of neutral protease stability (Imanaka *et al.*, 1986; Eijsink *et al.*, 1990, 1991b, 1992a,c,d;



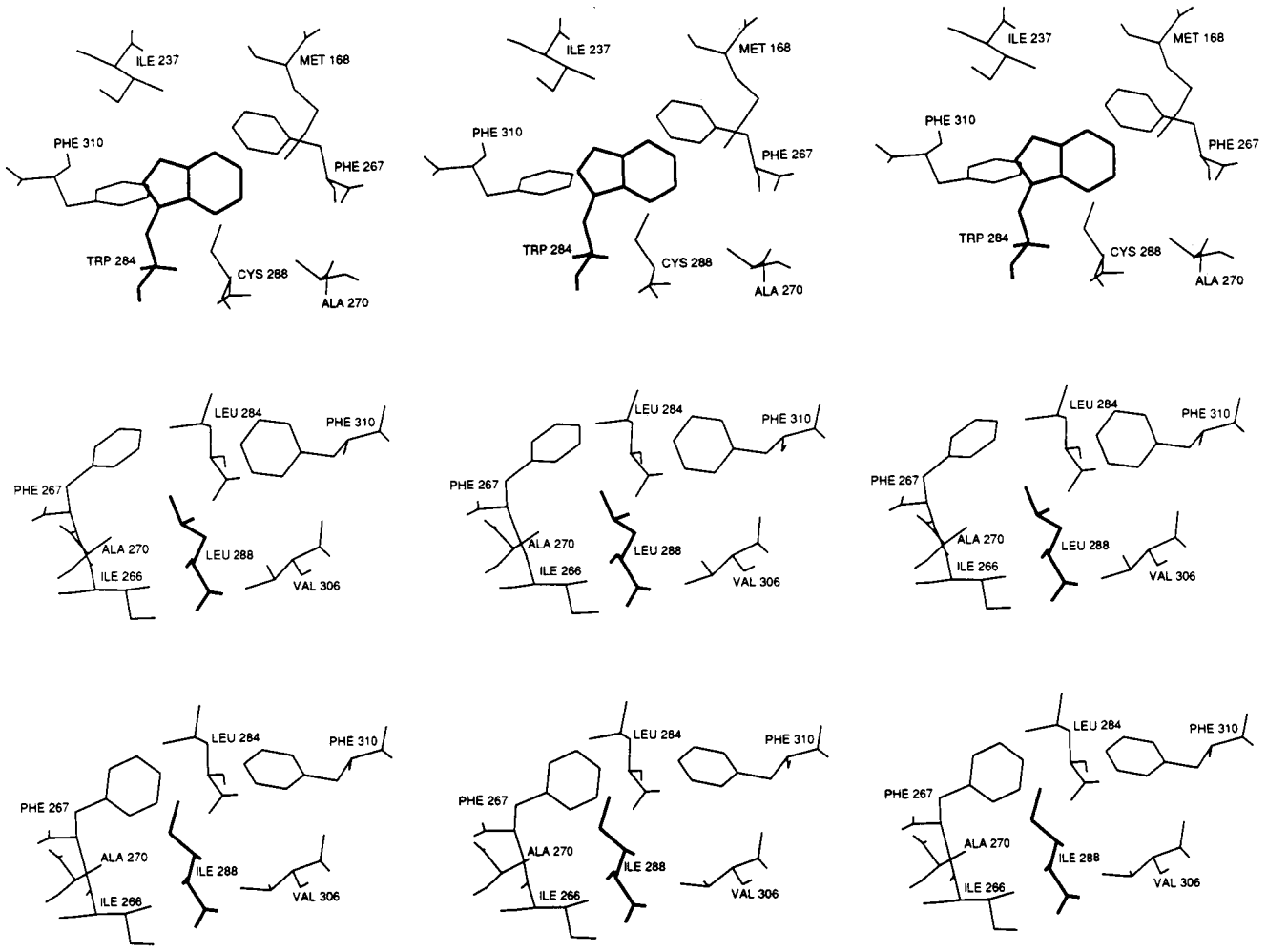


Fig. 4. Modelled structures of the environment of mutated residues. **(A)** Met168Trp, **(B)** Ala240Val, **(C)** Ala241Val, **(D)** Leu284Trp, **(E)** Cys288Leu, **(F)** Cys288Ile. Important characteristics of these structures are summarized in Table II.

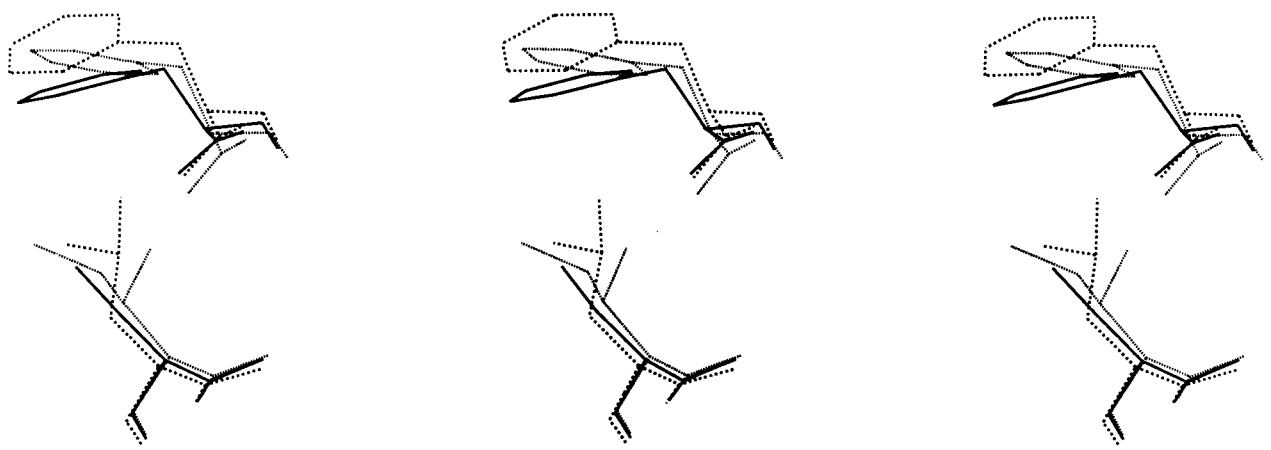


Fig. 5. The effect of the Cys288→Leu and Cys288→Ile substitutions on bending of Phe267. The Phe267 side chain bends away from the wild-type position (——) to accommodate Leu (· · · · ·) or Ile (·····) at position 288. Bending is more pronounced after Leu has been introduced because of Van der Waals overlaps involving the additional Cδ2 of Leu.

Toma *et al.*, 1991; Vriend *et al.*, 1991) clearly show that models built on the basis of the crystal structure of thermolysin are sufficiently accurate for the design of site-directed mutations. Therefore, despite the uncertainties related to model-building, it seems appropriate to conclude from the present data that mutations aimed at improving the packing density of the hydrophobic core are not an effective way to stabilize Nprs. Combining the present with previously described results, it would appear that mutational strategies for stabilization of Nprs should focus on the surface of the protein.

References

Aoki,T., Noguchi,N., Sasatsu,M. and Kono,M. (1987) *Gene*, **51,** 107–111.
Colman,P.M., Jansonius,J.N. and Matthews,B.W. (1972) *J. Mol. Biol.*, **70,** 701–724.
Conolly,M.L. (1985) *J. Am. Chem. Soc.*, **107,** 1118–1124.
Conolly,M.L. (1986) *Int. J. Peptide Protein Res.*, **28,** 360–363.
Creighton,T.E. (1991) *Curr. Opinion Struct. Biol.*, **1,** 5–16.
Dahlquist,F.W.,Long,J.W. and Bigbee,W.L. (1976) *Biochemistry*, **15,** 1103–1111.
Dill,K. (1990) *Biochemistry*, **29,** 7133–7155.
Eijsink,V.G.H., Vriend,G., Van den Burg,B., Venema,G. and Stulp,B.K. (1990) *Protein Engng*, **4,** 99–104.
Eijsink,V.G.H., Van den Burg,B., Vriend,G., Berendsen,H.J.C. and Venema,G. (1991a) *Biochem. Int.*, **24,** 517–525.
Eijsink,V.G.H., Van der Zee,J.R., Van den Burg,B., Vriend,G. and Venema,G. (1991b) *FEBS Lett.*, **282,** 13–16.
Eijsink,V.G.H., Vriend,G., Van der Zee,J.R., Van den Burg,B. and Venema,G. (1992a) *Biochem. J.*, **285,** 625–628.
Eijsink,V.G.H., Vriend,G., Van der Vinne,B., Hazes,B., Van den Burg,B. and Venema,G. (1992b) *Proteins*, **14,** in press.
Eijsink,V.G.H., Vriend,G., Van den Burg,B., Van der Zee,J.R., Veltman,O.R., Stulp,B.K. and Venema,G. (1992c) *Protein Engng*, **5,** 157–163.
Eijsink,V.G.H., Vriend,G., Van den Burg,B., Van der Zee,J.R. and Venema,G. (1992d) *Protein Engng*, **5,** 165–170.
Fujii,M., Takagi,M., Imanaka,T. and Aiba,S. (1983) *J. Bacteriol.*, **154,** 831–837.
Holmes,M.A. and Matthews,B.W. (1982) *J. Mol. Biol.*, **160,** 623–639.
Imanaka,T., Shibazaki,M. and Takagi,M. (1986) *Nature*, **324,** 695–697.
Jones,T.A. and Thirup,S. (1986) *EMBO J.*, **5,** 819–822.
Karpusas,M., Baase,W.A., Matsumura,M. and Matthews,B.W. (1989) *Proc. Natl Acad. Sci. USA*, **86,** 8237–8241.
Kauzman,W. (1959) *Adv. Protein Chem.*, **14,** 1–63.
Kellis, J.T.,Jr, Nyberg,K., Sali,D. and Fersht,A.R. (1988) *Nature*, **333,** 784–786.
Lim,W.A. and Sauer,R.T. (1989) *Nature*, **339,** 31–36.
Matsumara,M., Becktel,W.J. and Matthews,B.W. (1988) *Nature*, **334,** 406–410.
Matthews,B.W., Jansonius,J.N., Colman,P.M., Schoenborn,B.P. and Dupourque,D. (1972) *Nature New Biol.*, **238,** 37–41.
Mitchinson,C. and Wells,J.A. (1989) *Biochemistry*, **28,** 4807–4815.
Pakula,A.A. and Sauer,R.T. (1989) *Annu. Rev. Genet.*, **23,** 289–310.
Priest,F.G. (1989) In Harwood,C.R. (ed.), *Biotechnology Handbooks*, Vol. 2, *Bacillus*. Plenum Press, New York, pp. 293–320.
Rashin,A.A., Iofin,M. and Honig,B. (1986) *Biochemistry*, **25,** 3619–3625.
Sandberg,W.C. and Terwilliger,T.C. (1989) *Science*, **245,** 54–57.
Sandberg,W.C. and Terwilliger,T.C. (1991) *Proc. Natl Acad. Sci. USA*, **88,** 1706–1710.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74,** 643–657.
Sharp,K.A. (1991) *Curr. Opinion Struct. Biol.*, **1,** 171–174.
Shortle,D., Stites,W.E. and Meeker,A.K. (1990) *Biochemistry*, **29,** 8033–8041.
Stanssens,P., Opsomer,C., Mckeown,Y.M., Kramer,W., Zabeau,M. and Fritz,H.-J. (1989) *Nucleic Acids Res.*, **17,** 4441–4454.
Takagi,M., Imanaka,T. and Aiba,S. (1985) *J. Bacteriol.*, **163,** 824–831.
Tanford,C. (1980) *The Hydrophobic Effect*. Wiley, New York.
Toma,S., Campagnoli,S., Margarit,I., Gianna,R., Grandi,G., De Gregoriis,E. and Fontana,A. (1991) *Biochemistry*, **30,** 97–106.
Van den Burg,B., Eijsink,V.G.H., Stulp,B.K. and Venema,G. (1989) *J. Biochem. Biophys. Methods*, **18,** 209–220.
Van den Burg,B., Enequist,H.G., Van der Haar,M.E., Eijsink,V.G.H., Stulp,B.K. and Venema,G. (1991) *J. Bacteriol.*, **173,** 4107–4115.
Van Gunsteren,W.F. and Berendsen,H.J.C. (1987) BIOMOS, Biomolecular Software, Laboratory of Physical Chemistry, University of Groningen, Groningen, The Netherlands.
Voorintholt,R., Kosters,M.T., Vegter,G., Vriend,G. and Hol,W.G.J. (1989) *J. Mol. Graphics*, **7,** 243–245.
Vriend,G. (1990) *J. Mol. Graphics*, **8,** 52–56.
Vriend,G., Berendsen,H.J.C., Van der Zee,J.R., Van den Burg,B., Venema,G. and Eijsink,V.G.H. (1991) *Protein Engng*, **4,** 941–945.
Yutani,K., Ogashara,K., Tsujita,T. and Sugino, Y. (1987) *Proc. Natl Acad. Sci. USA* **84,** 4441–4444.
Zell,R. and Fritz,H.-J. (1987) *EMBO J.*, **6,** 1809–1815.

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Note added in proof

Recently we found that the replacement of the bulky hydrophobic residues in the interior of the Npr-ste by alanine had only marginal effects on the thermostability, confirming the insensitivity of the Npr-ste towards mutations in its hydrophobic core.